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Oral vaccination with influenza hemagglutinin combined with human pulmonary surfactant-mimicking synthetic adjuvant SF-10 induces efficient local and systemic immunity compared with nasal and subcutaneous vaccination and provides protective immunity in mice

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ABSTRACT

We reported previously that a synthetic mucosal adjuvant SF-10, which mimics human pulmonary surfactant, delivers antigen to mucosal dendritic cells in the nasal cavity and promotes induction of humoral and cellular immunity. The aim of the present study was to determine the effects of oral administration of antigen combined with SF-10 (antigen-SF-10) on systemic and local immunity. Oral administration of ovalbumin, a model antigen, combined with SF-10 enhanced ovalbumin uptake into intestinal antigen presenting MHC II^+CD11c^+ cells and their $CD11b^+CD103^+$ and $CD11b^+CD103^-$ subtype dendritic cells, which are the major antigen presenting subsets of the intestinal tract, more efficiently compared to without SF-10. Oral vaccination with influenza hemagglutinin vaccine (HAv)-SF-10 induced HAv-specific IgA and IgG in the serum, and HAv-specific secretory IgA and IgG in bronchoalveolar lavage fluid, nasal washes, gastric extracts and fecal material; their levels were significantly higher than those induced by subcutaneous HAv or intranasal HAv and HAv-SF-10 vaccinations. Enzyme-linked immunospot assay showed high numbers of HAv-specific IgA and IgG antibody secreting cells in the gastrointestinal and respiratory mucosal lymphoid tissues after oral vaccination with HAv-SF-10, but no or very low induction following oral vaccination with HAv alone. Oral vaccination with HAv-SF-10 provided protective immunity against severe influenza A virus infection, which was significantly higher than that induced by HAv combined with cholera toxin. Oral vaccination with HAv-SF-10 was associated with unique cytokine production patterns in the spleen after HAv stimulation; including marked induction of HAv-responsive Th17 cytokines (e.g., IL-17A and IL-22), high induction of Th1 cytokines (e.g., IL-2 and IFN-γ) and moderate induction of Th2 cytokines (e.g., IL-4 and IL-5). These results indicate that oral vaccination with HAv-SF-10 induces more efficient systemic and local immunity than nasal or subcutaneous vaccination with characteristically high levels of secretory HAv-specific IgA in various mucosal organs and protective immunity.

1. Introduction

Influenza virus infects approximately 10–20% of the world population annually, causing death of approximately 290,000 to 650,000 individuals [1]. Since several types of influenza virus spread globally among humans as well as pets and other domestic animals [2–5], there is a need for effective influenza vaccines for both human and animals. In particular, induction of protective immunity in the respiratory and gastrointestinal mucosae is important for protection against influenza virus infection, because seasonal influenza viruses grow in the respiratory mucosa and the highly pathogenic avian influenza viruses grow in both the respiratory and gut mucosae [6–8].

The currently available influenza vaccines administered intramuscularly or subcutaneously induce a predominant IgG-mediated protection in the systemic immune compartment [9], but hardly induce adequate production of antiviral secretory IgA (S-IgA) for protection against infection in the airway mucosa, the site of viral entry and propagation. To overcome this shortfall, previous studies investigated the benefits of intranasal mucosal vaccine adjuvant, which can induce both S-IgA in the airway mucosa and IgG in serum [9–12]. However, many such vaccines were found ineffective or had safety problems [11,13]. We reported previously

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that pulmonary surfactant, Sufracten®, was effective and safe intranasal mucosal adjuvant for influenza ether split hemagglutinin vaccine (HAv) and showed potent enhancement of humoral immune responses without adverse reactions in the mouse and swine [11,14]. Pulmonary surfactant shows potent adjuvanticities as a delivery vehicle by promoting antigens uptake into mucosal antigen presenting cells (APCs), such as dendritic cells (DCs), but itself does not stimulate APCs without antigen [11,15]. Among the various constituents of human pulmonary surfactant, we found three major lipids; 1,2-dipalmitoyl-phosphatidylcholine (DPPC), phosphatidylglycerol (PG), and palmitic acid (PA), together with surfactant protein C (SP-C), to be essential constituents for its mucosal adjuvanticity [15]. We also prepared a synthetic surfactant (SSF) of these constituents that mimics the antigen delivery activity of pulmonary surfactant. Furthermore, we recently improved the SSF for large-scale manufacturing and enhancement of its adjuvanticity [12].

Since the hydrophobic peptide SP-C does not dissolve in common organic solvents, we selected K6L16 peptide among the SP-C related peptides, as a substitute for human SP-C, which is soluble in methanol and ethanol and expresses high adjuvanticity [12]. To achieve more efficient antigen delivery activity for SSF, we added carboxy vinyl polymer (CVP) to increase the viscosity of antigen-SSF complex and prolong antigen uptake time on the mucosal surface. Based on these improvements, the manufactured pulmonary surfactant mimicked the synthetic adjuvant, which is useful for ample supply and shows enhanced adjuvanticity, and termed the improved adjuvant compound SF-10 [12]. Antigen combined with SF-10 (antigen-SF-10) enhances absorption of antigens into nasal APCs and induces systemic and respiratory humoral immunity with induction of balanced antigen specific Th1/Th2 cells in mice and cynomolgus monkeys [12,16] and systemic cell-mediated immunity as well in mice [17].

The pulmonary surfactant is a biologically active natural substance secreted by alveolar type II epithelial cells, rapidly metabolized in the lungs, coats the alveolar surface, and lowers the surface tension of the air-liquid interface to prevent alveolar collapse [18]. Previous studies indicated a progressive increase in micelles of pulmonary surfactant in human amniotic fluid at term and that the micelles in the amniotic fluid are swallowed by the fetus [19,20]. More recently, Nishijima et al. [21] reported that the micelles swallowed by the fetus are absorbed by the intestinal mucosal epithelium where they stimulate villous height maturation. These findings suggest that pulmonary surfactant has a physiological function in the gastrointestinal tract apart from its function in the respiratory tract through efficient absorption by the intestinal mucosal epithelium. Accordingly, it is assumed that effective antigen presentation in the gastrointestinal mucosal APCs can be achieved by oral vaccination of antigen-SF-10 complex through the delivery activity of pulmonary surfactant to the gastrointestinal mucosal epithelium.

Although oral vaccination could result in gastrointestinal antigen digestion and acid inactivation of the antigen, compared with nasal vaccination, various oral influenza vaccines, combined with adjuvants, such as heat-labile enterotoxin-based adjuvants [22,23], liposome-based adjuvant [24], and influenza DNA vaccines using adenovirus vector [25,26], plasmid vector [27], and Salmonella vector [28,29] have been used. Although these oral influenza vaccines require higher doses of antigen by several orders of magnitude [30], the level of induced immunity was relatively lower than that achieved through nasal and subcutaneous vaccinations. In this regard, whereas oral administration of DNA vaccines can reduce vaccine doses, it carries the risk of releasing genetically modified organisms to nature.

The aim of the present study was to determine the effects of oral administration of HAv-SF-10 into the gastrointestinal tract of mice on respiratory, gastrointestinal and systemic immunity. For this purpose, we analyzed the induction of HAv-specific antibody secreting cells (ASCs) in the respiratory and gut organ-associated lymphoid tissues, and the levels of cytokines secreted by HAv responsive helper T cells in the spleen of immunized mice to evaluate the Th1/Th2/Th17 immunity balance. We also assessed the level of protective immunity induced by oral administration of HAv-SF-10 against severe influenza A virus (IAV) challenge in a mouse model of acute pneumonia.

2. Materials and methods

2.1. Vaccine, virus and animals

Split product of HAv of IAV/California/7/2009(H1N1) was purchased from Kitasato Daiichi Sankyo Vaccine Co. (Tokyo, Japan). IAV/ PR8/34(H1N1) was provided by The Research Foundation for Microbial Diseases of Osaka University (Kagawa, Japan). Female BALB/c mice (age 6–8 weeks) were purchased from Japan SLC (Shizuoka, Japan). All animals were maintained under specific-pathogen-free conditions. Mice were anesthetized by intraperitoneal injection of 62.6 mg ketamine and 12.4 mg xylazine per kg body weight before vaccine administration and infection. All mice used in this study were treated according to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, 1996). The study was approved by the Animal Care Committee of Tokushima University (#T27-109).

2.2. Preparation of antigen-SF-10

The procedure for preparation of SSF was reported in detail previously [15]. Briefly, SSF was prepared by mixing three major lipids of pulmonary surfactant, DPPC, PG and PA, and a cationic hydrophobic peptide K6L16, which mimics SP-C, at a weight ratio of 75:25:10:2. SSF was mixed with HAv or Alexa647-labeled ovalbumin (fOVA, Thermo Fisher Scientific, Middletown, VA) at a SSF phospholipid:antigen protein ratio of 10:1 at 42 °C, and the stable antigen-SSF complex was prepared by lyophilization and stored at −20 °C until use. To prepare 1% CVP (HIVISWAKO104, FUJIFILM Wako Pure Chem. Co., Osaka, Japan) solution, CVP powder was dissolved in saline by stirring followed by sonication and then stored at 4 °C until use. Before administration, lyophilized antigen-SSF was dissolved with 1% CVP by mild mixing and then equal volume of saline [12] or 50 mM sodium bicarbonate buffer, pH 9.7, (carbo-buffer) was added. The final solution in the presence of CVP was termed antigen-SF-10.

2.3. Immunization and sampling of serum and mucosal fluid

After starvation for 4 h, one group of mice were orally administered 1 μg HAv with or without 10 μg phospholipid-containing SF-10 [HAv-SF-10(p.o.)], lipopolysaccharide (LPS) (Nacalai tesque, Kyoto, Japan), Pam3CSK4 (InvivoGen, San Diego, CA), poly(I:C) (Alexis Biochemicals, Lausanne, Switzerland), CpG (InvivoGen), cholera toxin B subunit (CT-B) (Sigma-Aldrich, St. Louis, MO), or cholera toxin (CT) (Millipore, Billerica, MA) in 200 μL of 25 mM carbo-buffer by using a stainless steel mouse feeding needle (Fuchigami Co., Ltd., Kyoto, Japan, No. 4202, L × diam. 38 × 0.62 mm), connected to a 1 mL plastic syringe, four times vaccination at days 0, 3, 14, and 17 or at the indicated time points of vaccination schedule described in the legends for figures. Another group of mice was inoculated intranasally with 1 μg HAv with or without 10 μg SF-10 in 6 μL of saline (3 μL/each nostril) [HAv-SF-10(i.n.)] by using a Eppendorf® Research® plus pipette, Z683787 Aldrich or subcutaneously injected with 1 μg HAv in 100 μL saline

[HAv(s.c.)] by using a 1 mL plastic syringe at days 0, 3, 14, and 17, respectively. After 2 weeks from the last inoculation, serum, nasal washes, and bronchoalveolar lavage fluids (BALF) were prepared as described previously [11]. The gastric extract was prepared by allowing the stomach to stand in a 2% saponin (Nacalai tesque)-PBS overnight at 4 °C. Fecal extract was prepared by extraction of 100 mg feces from the cecum with 500 μL phosphate-buffered saline (PBS) containing protease inhibitors, e.g., 0.32 mg/mL benzamide (Sigma-Aldrich) and 2 μg/mL phenylmethylsulfonyl fluoride (Nacalai tesque).

2.4. Measurement of antigen uptake by small intestinal mucosal cells

Mice were orally administrated of 100 μg fOVA with or without SF-10. At 12 or 24 h after vaccination, small intestinal mucosal cells were isolated, and the epithelial layer cells and lamina propria layer cells were purified separately by 2 mM EDTA (Nacalai tesque) solution and 1.5 mg/mL collagenase type IV (Gibco, Grand Island, NY) in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution, respectively, as described in detail previously [31]. These cells were stained with anti-mouse I-A/I-E (MHC II), CD11b, CD11c and CD103 (BioLegend, San Diego, CA) and analyzed by FACSVerse (BD Biosciences, Franklin Lakes, NJ) and Flowjo software (Tree Star Inc., Ashland, OR).

2.5. Measurement of HAv-specific IgA, S-IgA and IgG

Two weeks after the last immunization, we measured HAv-specific IgA and IgG levels in the serum and HAv-specific S-IgA and IgG levels in the mucosal fluid by enzyme-linked immunosorbent assay (ELISA), as described previously [15]. We used goat anti-mouse IgA and anti-mouse IgG antibody conjugated with horseradish peroxidase (Sigma-Aldrich) as the secondary antibodies. HAv-specific IgA, S-IgA and IgG levels were calculated by using affinity-purified mouse HAv-specific IgA and IgG as standards, as described previously [11].

2.6. Detection of HAv-specific antibody secreting cells in lymph nodes by ELISPOT assay and measurement of HAv responsive cytokine levels in the spleen

Two weeks after the fourth oral vaccination, the spleen, cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), Peyer's patches (PP), gastric lymph nodes (GLN), and lung lymph nodes were isolated from mice immunized with HAv or HAv-SF-10 in carbo-buffer. The lymphocytes from the spleen, CLN, PP, MLN, and GLN were purified according to the method described previously [17] while those from lung were purified by collagenase digestion followed by Percoll (GE Healthcare, Buckinghamshire, UK) density gradient centrifugation, as described previously [17].

For detection of HAv-specific ASCs by enzyme-linked immunospot (ELISPOT) assay, the isolated lymphocytes from each organ, except the lung, were incubated for 3 days with 1 μg/mL R848 (Novus Biologicals, Littleton, CO) and 10 ng/mL rmIL-2 (BioLegend) in complete RPMI (cRPMI) medium (containing 10 mM HEPES, pH 7.2, 1 mM sodium pyruvate, 1% MEM non-essential amino acids solution, 14.3 μM 2-mercaptoethanol, 10 μg/mL gentamycin and 10% heat-inactivated fetal bovine serum). The incubated lymphocytes and non-incubated lung lymphocytes isolated from mice immunized orally with HAv or HAv-SF-10 were counted by LUNA-II™ Automated Cell Counter (Logos Biosystems, Anyang, Korea). The lymphocytes were then seeded at the indicated number (from 10^5 to 10^6 cells/well) onto MultiScreen 96-well plates MSIPS4510 (Millipore), which were pre-coated with 1 μg HAv protein/well, and incubated with fresh cRPMI for 4 h (lung lymphocytes) or 24 h (excluding lung lymphocytes). After incubation, the spots of HAv-specific ASCs were detected by goat anti-mouse IgA or anti-mouse IgG antibody conjugated with horseradish peroxidase (Sigma-Aldrich) and 3-amino-9-ethylcarbazole (Sigma-Aldrich). The number of ASC spots was counted by ELISPOT counter ImmunoSpot S6 (CTL, Cleveland, OH).

For measurement of cytokine levels secreted by HAv-responsive splenocytes of mice immunized subcutaneously, intranasally or orally with HAv or HAv-SF-10, isolated splenocytes were incubated with cRPMI medium with or without 10 μg HAv per 10^6 cells/mL for 3 days. The concentration of IL-2, IL-4, IL-5, IL-17A, IL-21 and IFN-γ in the culture media supernatant was measured by LEGENDplex™ and analyzed by LEGENDplex™ Data Analysis Software (BioLegend) according to the protocols provided by the manufacturers.

2.7. Hemagglutination inhibition assay

Hemagglutination inhibition (HI) activity was measured as described previously [14]. Serum was treated overnight with RDE (II) "SEIKEN" (Denka Seiken Co., Tokyo, Japan) at 37 °C. The treated serum was diluted in 96-well plates, and then 4 HA units of the vaccine antigen and 0.75% of washed chicken red blood cells were added to each well, and incubated for 30 min at room temperature. The HI endpoint titers represented the reciprocal of the highest serum dilution that produced complete inhibition of hemagglutinating activity.

2.8. Protection against virus challenge in mice after oral vaccination

To assess the efficacy of protective immunity after oral vaccination, BALB/c mice were immunized twice orally with HAv, HAv-SF-10 or carbo-buffer at days 0 and 3, and then all mice were infected intranasally with 20 μL of IAV/ PR8/34(H1N1) at a dose of $50 \times LD_{50}$ (50 PFU) at 14 days after the last immunization. At days 4 and 7 after infection, the virus titers in BALF were measured by the plaque assay using Madin-Darby canine kidney cells and anti-IAV nucleoprotein monoclonal antibody, as described previously [32].

Another group of BALB/c mice was immunized twice orally with HAv, HAv-SF-10 or HAv-CT at days 0 and 3, and then all mice were infected intranasally with 20 μL of a lethal dose of IAV/ PR8/34(H1N1) (50 to 1000 PFU), and the survival and body weight loss were monitored during the period of 15 days after infection.

2.9. Statistical analysis

Data were expressed as mean ± SEM. Statistical significance of the differences between experimental groups was calculated by ANOVA with a Bonferroni posttest, an unpaired Student's *t*-test (if variances were equal (*F*-test, $P \geq 0.05$)) or Welch's *t*-test (if variances were unequal (*F*-test, $P < 0.05$)) using KaleidaGraph 4.5 software (Synergy Software, Reading, PA). Survival rate was analyzed by the Kaplan-Meier and log-rank tests. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. SF-10 increases antigen uptake into intestinal APCs

To assess the effects of SF-10 on antigen uptake into intestinal APCs, mice were orally administrated reporter antigen fOVA and fOVA-SF-10 for 12 h and 24 h, and APCs in the epithelial and lamina propria layers of the small intestine were isolated. fOVA-labelled MHC II^+CD11c^+ cells (APCs), and $CD11b^+CD103^+$ and $CD11b^+CD103^-$

DC subtypes, which are the major DC subtypes in APCs [33], were detected by flow cytometry (Fig. 1A-E). The percentages of fOVA-labelled cells among the APCs (Fig. 1F), $CD11b^{+}CD103^{+}$ DCs (Fig. 1G) and $CD11b^{+}CD103^{-}$ DCs (Fig. 1H) in the epithelial and lamina propria layers indicated that SF-10 significantly increased fOVA-uptake into the intestinal APCs after administration for 12 h, compared with those induced by fOVA alone. However, the percentages of fOVA-labelled cells in APCs were markedly decreased after administration for 24 h and no significant differences were detected between fOVA and fOVA-SF-10 treated mice. These results indicate that SF-10 adjuvant enhances antigen uptake into APCs in the gastrointestinal mucosa for at least 12 h.

3.2. Oral vaccination of HAv-SF-10 induces higher levels of HAv-specific IgA and IgG levels in serum and HAv-specific S-IgA and IgG in various mucosal fluids compared with nasal and subcutaneous vaccination and increases HAv-specific IgA and IgG ASCs in various lymph nodes throughout the body

To compare the effects of oral vaccination of HAv-SF-10 on the induction of humoral immune responses with other administration routes of vaccination, mice were immunized orally, intranasally and subcutaneously 4 times with the same administration doses of HAv and HAv-SF-10. The serum, BALF, nasal washes, and gastric and fecal extracts were prepared at 2 weeks after the last immu-

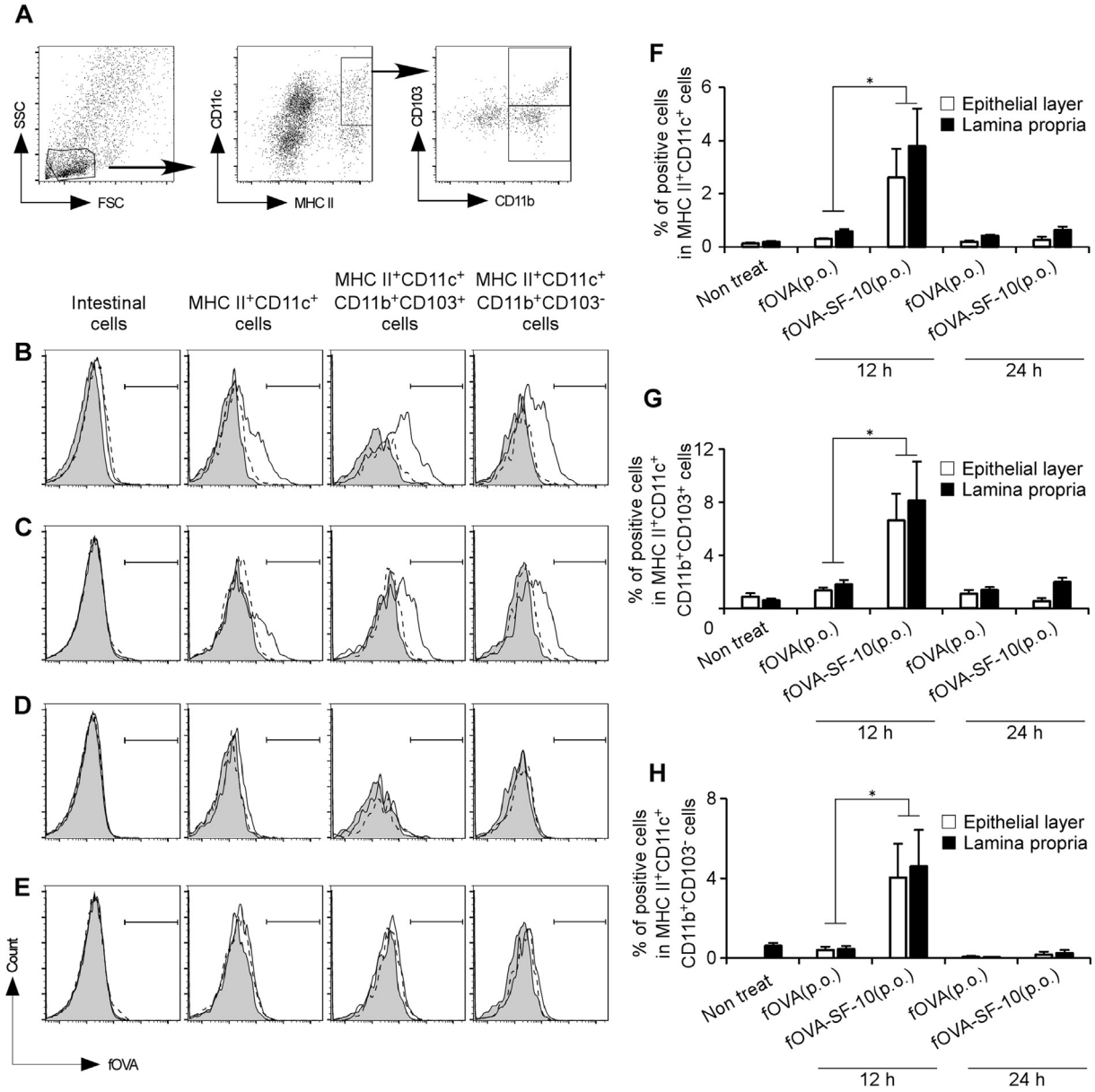

Fig. 1. SF-10 enhances antigen uptake by intestinal mucosal APCs. BALB/c mice was administrated fOVA or fOVA-SF-10 orally. At 12 h (B, C) or 24 h (D, E) after administration, the mucosal epithelial layer cells (B, D) and lamina propria layer cells (C, E) of the small intestine were isolated. fOVA uptake into the intestinal cells, such as MHC $II^{+}CD11c^{+}$ APCs, and $CD11b^{+}CD103^{+}$ and $CD11b^{+}CD103^{-}$ DCs, was analyzed by flow cytometry. (A) The gating strategy of each cell population. (B-E) fOVA uptake into each type of cell from non-treated mice (gray), fOVA- (dashed line) and fOVA-SF-10- (solid line) immunized mice. (F-H) Percentages of fOVA incorporated cells in MHC $II^{+}CD11c^{+}$ APCs (F), $CD11b^{+}CD103^{+}$ DCs (G), and $CD11b^{+}CD103^{-}$ DCs (H). Data are mean ± SEM and were analyzed for statistical significance by using independent Student's *t*-test or Welch's *t*-test (n = 5). $^{*}P < 0.05$. Experiments were independently repeated two times with 5 mice per group.

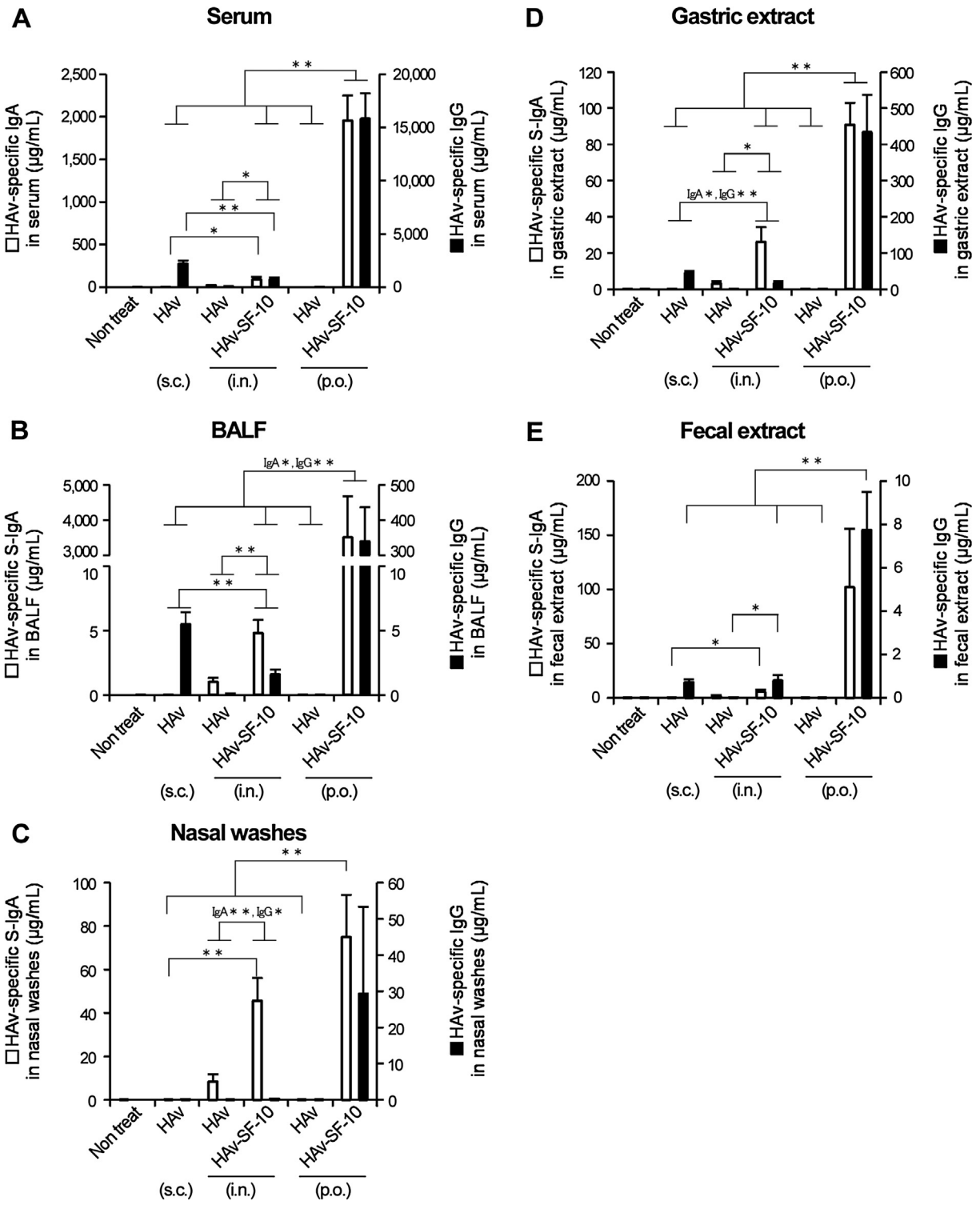

Fig. 2. Comparison of the effects of immunization with HAv-SF-10(p.o,), HAv-SF-10(i.n.), HAv(p.o.), HAv(i.n.) or HAv(s.c.) on mucosal and systemic immunity. Mice were immunized four times by intranasal (i.n.) or oral administration (p.o.) of HAv combined with or without SF-10 at days 0, 3, 14, and 17. Another group of mice were immunized with HAv subcutaneously (s.c.) at the same time schedules. At 2 weeks after the last immunization, the levels of HAv-specific IgA and IgG antibodies in serum (A) and HAv-specific S-IgA and IgG antibodies in BALF (B), nasal washes (C), gastric extract (D) and fecal extracts (E) were measured by ELISA. Data are mean ± SEM of IgA or S-IgA (open column) and IgG (closed column) of 6 mice and statistical significance was analyzed by using ANOVA with a Bonferroni posttest. $^{*}P < 0.05$, $^{**}P < 0.01$. Experiments were independently repeated two times with 6 mice per group.

nization, and the HAv-specific IgA, S-IgA and IgG antibodies of these specimens were measured by ELISA (Fig. 2). Administration of HAv alone without SF-10, either intranasally [HAv(i.n.)] or orally [HAv(p.o.)], was associated with very low levels of HAv-specific IgA, S-IgA and IgG in all specimens. HAv(s.c.) induced only moderate levels of HAv-specific IgG but not S-IgA and IgA in all specimens. HAv-SF-10(i.n.) was associated with significantly higher levels of HAv-specific IgA, S-IgA and IgG in all specimens compared

with the levels of HAv(i.n.). In contrast, HAv-SF-10(p.o.) was associated with noticeable induction levels of both IgA and IgG in serum at about 20-fold higher than those by HAv-SF-10(i.n.) (Fig. 2A) and S-IgA and IgG in BALF at about 700- and 200-fold higher than those by HAv-SF-10(i.n.), respectively (Fig. 2B). Induction of HAv-specific IgG by HAv-SF-10(p.o.) was also significantly higher at about 7-fold that by HAv(s.c.) (Fig. 2A). The induced levels of HAv-specific S-IgA and IgG in nasal washes (Fig. 2C), gastric extracts (Fig. 2D) and fecal extracts (Fig. 2E) of mice immunized with HAv-SF-10(p.o.) were significantly higher at about 2- to 20-fold and 10- to 50- fold, respectively, than those by HAv-SF-10(i.n.).

Table 1
Serum HI titer after 4 times vaccination.

	HI titer
Non treatment	<10
HAv (subcutaneously)	1493 ± 213
HAv (intranasally)	30 ± 7
HAv-SF-10 (intranasally)	137 ± 59
HAv (orally)	6 ± 3
HAv-SF-10 (orally)	3307 ± 849

mean ± SEM (n = 6)

The mean HI titers in serum of mice after vaccination 4 times with HAv-SF-10(p.o.) were the highest at 3307 ± 849 among the vaccination groups tested (Table 1): 2.2-fold of HAv(s.c.), 24-fold of HAv-SF-10(i.n.), and those in mice treated with HAv(p.o.) and HAv(i.n.) were < 40.

To confirm the production sites of HAv-specific IgA, S-IgA and IgG in mice vaccinated orally 4 times with HAv or HAv-SF-10, we isolated lymphocytes from the lung, spleen, CLN, MLN, PP and GLN at 2 weeks after the last immunization, and measured the HAv-specific IgA and IgG ASCs by ELISPOT assay. Both HAv-specific IgA and IgG ASCs were detected in all lymphocytes of lung, spleen, CLN, MLN, PP and GLN of mice immunized with HAv-SF-10 (p.o.), but not those immunized with HAv(p.o.) alone, except detection of few IgG spots in CLN and spleen (Fig. 3). The numbers of induced HAv-specific IgA and IgG spots of ASCs in the lung of mice

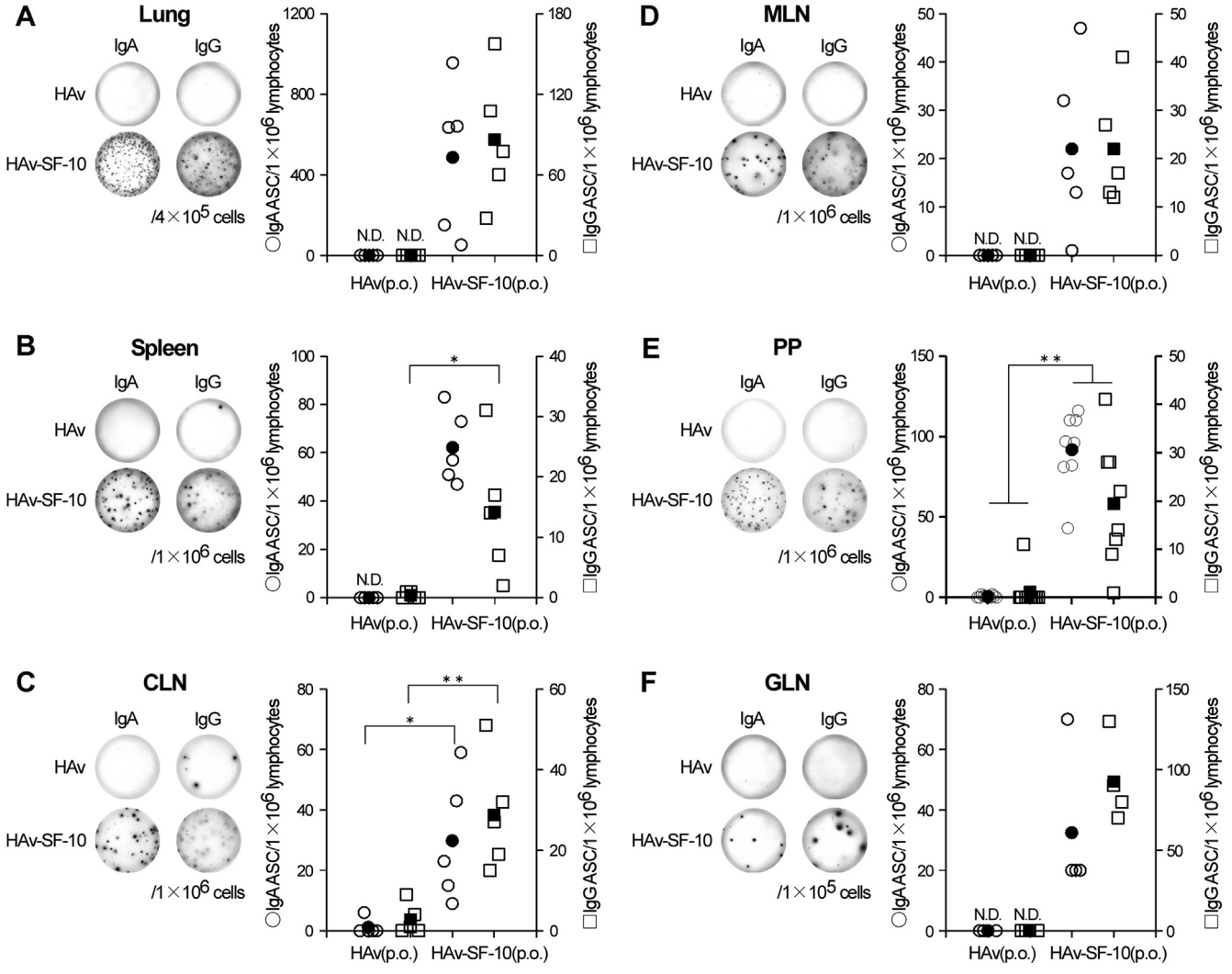

Fig. 3. Detection of HAv-specific ASCs in respiratory and gastrointestinal mucosal tissues. Mice were immunized four times with HAv-SF-10(p.o.) or HAv(p.o.) at days 0, 3, 14, and 17. At 2 weeks after the last immunization, lymphocytes from the lungs (A), spleen (B), cervical lymph nodes (CLN) (C), mesenteric lymph nodes (MLN) (D), Peyer's patches (PP) (E) and gastric lymph nodes (GLN) (F) were isolated. They were then seeded onto HAv-coated plates at the indicated number of cells, and HAv-specific ASCs were detected by ELISPOT. Data represent HAv specific S-IgA (circles), IgG (squares) and numbers of ASC per 10^6 cells of individuals (open) and their means (solid) (n = 5–10). N.D.: not detected. Experiments were independently repeated two times with 5–10 mice per group and statistical significance was analyzed by using Welch's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

immunized with HAv-SF-10(p.o.) were the highest among those of ASCs in the lymph nodes throughout the body analyzed.

3.3. Induction of HAv-specific Th1, Th2 and Th17-type immune responses following oral vaccination with HAv-SF-10

To analyze the immunological background of high induction levels of HAv-specific IgG and IgA in serum and HAv-specific S-IgA in mucosal fluid of mice vaccinated with HAv-SF-10(p.o.), we measured cytokine induction of HAv-specific Th1, Th2 and Th17-type immune responses of splenocytes. Mice were immunized four times with HAv(i.n.), HAv(p.o.), HAv(s.c.), HAv-SF-10(i.n.) and HAv-SF-10(p.o.), and the splenocytes of these mice were isolated at 2 weeks after the last immunization, followed by incubation with or without HAv for 3 days. The HAv responsive Th1 (IL-2 and IFN-γ), Th2 (IL-4 and IL-5) and Th17 (IL-17A and IL-22) cytokine levels in culture media were measured (Table 2). In the absence of HAv in the culture medium, no significant induction of cytokines was detected compared with those of no-treatment control in all the vaccination groups. HAv stimulated the induction of all cytokines in each group of immunized mice. HAv(s.c.) vaccination induced both Th1 and Th2 cytokines, and the levels of IL-4 and IL-5 were the highest among the immunized groups. In contrast, HAv-SF-10(p.o.) and HAv-SF-10(i.n.) vaccinations mainly increased the levels of IFN-γ, IL-2, IL-17A and IL-22, compared with HAv(p.o.) and HAv(i.n.), respectively, and the production levels were higher by HAv-SF-10(p.o.) than by HAv-SF-10(i.n.). These results indicate that HAv-SF-10(p.o.) vaccination markedly increases Th1 and Th17 cytokine production with moderate Th2 cytokine induction, whereas Th17 cytokine induction by HAv-SF-10(p.o.), in particular, is the highest among the immunized groups tested.

3.4. Oral vaccination time schedule and comparison of induction efficacy of HAv-specific IgG in serum and S-IgA in BALF among various mucosal adjuvants

To optimize the time of oral vaccination, HAv-SF-10(p.o.) was administered one to four times, as illustrated in Fig. 4A. The levels of HAv-specific IgG in serum and HAv-specific S-IgA in BALF were measured at 2 weeks after the last immunization. Serum IgG and BALF S-IgA increased exponentially with time of vaccination and serum HAv-specific IgG levels reached about 500 μg/mL after two times immunization, the levels were almost equivalent to the IgG titers with complete protection against severe IAV infection, which was induced in mice administrated with HAv-SF-10(i.n.), as reported previously [12]. These data suggest that two-time vaccination with HAv-SF-10(p.o.) can provide protective immunity.

We next compared the induction efficacy of SF-10 and other mucosal adjuvants on HAv-specific IgG in serum and S-IgA in BALF. Mice were immunized twice orally with HAv or HAv-SF-10, -LPS, -Pam3CSK4, -poly(I:C), -CpG, -CT-B, or –CT. Two weeks after the last immunization, the levels of HAv-specific IgG in serum and S-IgA in BALF were measured by ELISA (Fig. 4C). No significant induction of HAv-specific IgG in serum and S-IgA in BALF was noted in mice immunized orally with HAv-LPS, -Pam3CSK4, -poly(I:C), -CpG, and -CT-B, compared with immunization using HAv alone. Among the tested adjuvants derived from bacteria and virus origins, only CT with HAv [HAv-CT(p.o.)] was associated with induction of HAv-specific IgG in serum and very low levels of HAv-specific S-IgA in BALF, but the levels were significantly lower than those of mice immunized with HAv-SF-10(p.o.). The induced levels of HAv-specific IgG by HAv-SF-10(p.o.) were 5.5-fold higher in serum and HAv-specific S-IgA were 63-fold higher in BALF, compared with those induced by HAv-CT(p.o.).

3.5. Protective immunity induced by oral vaccination of HAv-SF-10

Finally, we analyzed the protective immunity induced by two-time vaccination with HAv-SF-10(p.o.), by comparing HI titers in serum with those of HAv(p.o.) and HAv-CT(p.o.) (Fig. 5A). The induced HI titer of HAv-SF-10(p.o.) was > 160, which was significantly higher than that of HAv-CT(p.o.).

Fig. 5B shows the neutralization activities of BALF in mice immunized with HAv-SF-10(p.o.), HAv(p.o.) and carbo-buffer (negative control). Immunized mice were infected with IAV/PR8(H1N1) at a lethal dose of 50 PFU ($50 \times LD_{50}$) at 2 weeks after the last immunization and then virus titers in BALF were measured at days 4 and 7 after infection. More than 10^4 PFU/mL of IAV was detected in BALF of mice immunized with carbo-buffer and HAv(p.o.) at days 4 and 7, but only 20 PFU/mL at day 4 and below the detection levels at day 7 were detected in the BALF of mice immunized with HAv-SF-10(p.o.).

We also monitored the survival rates (Fig. 5C) and changes in body weight (Fig. 5D) of mice immunized with HAv, HAv-CT, or HAv-SF-10 after infection with three lethal doses of IAV/PR8 (H1N1) (50, 250, and $1000 \times LD_{50}$) over a period of 15 days. All mice that were immunized with HAv(p.o.) died at day 9 after infection with $50 \times LD_{50}$ IAV/PR8(H1N1) and only 20% of mice immunized with HAv-CT(p.o.) survived. In contrast, all mice immunized with HAv-SF-10(p.o.) survived following infection with $50 \times LD_{50}$ IAV/PR8(H1N1) ($P = 0.004$, vs. HAv(p.o.); $P = 0.084$, vs. HAv-CT(p.o.)) and also following infection with $250 \times LD_{50}$ and $1000 \times LD_{50}$ IAV/PR8(H1N1). Mice immunized with HAv-SF-10(p.o.) showed rapid recovery of body weight loss following infection with $50 \times LD_{50}$ IAV/PR8(H1N1), but recovery delay was detected after 250 and $1000 \times LD_{50}$ IAV/PR8(H1N1) infection.

4. Discussion

The present study describes several new findings on pulmonary surfactant mimicking adjuvant SF-10. The findings include i) Administration of antigen-SF-10(p.o.) enhanced antigen uptake

Table 2
Cytokine concentrations (pg/mL) in the culture media of splenocytes stimulated with (+) or without (-) HAv.

	Th1				Th2				Th17			
	IL-2		IFN-γ		IL-4		IL-5		IL-17A		IL-22	
HAv stimulation	–	+	–	+	–	+	–	+	–	+	–	+
Non treatment	82 ± 34	40 ± 2	10 ± 5	23 ± 6	1 ± 1	0 ± 0	0 ± 0	0 ± 0	8 ± 8	3 ± 3	36 ± 18	35 ± 7
HAv (subcutaneously)	59 ± 19	1608 ± 117*	28 ± 14.1	2538 ± 607*	1 ± 1	188 ± 38**	0 ± 0	2143 ± 458*	8 ± 7	60 ± 31**	57 ± 29	562 ± 153**
HAv (intranasally)	69 ± 26	547 ± 183**	13 ± 6	575 ± 262**	0 ± 0	5 ± 2*	0 ± 0	64 ± 23	1 ± 1	93 ± 70**	59 ± 29	250 ± 87**
HAv-SF-10 (intranasally)	75 ± 8	2445 ± 584	8 ± 4	648 ± 298**	0 ± 0	6 ± 1*	0 ± 0	70 ± 20	0 ± 0	1469 ± 484**	38 ± 21	1026 ± 364**
HAv (orally)	57 ± 9	61 ± 22*8	6 ± 3	16 ± 7**	0 ± 0	1 ± 0**	0 ± 0	1 ± 1*	0 ± 0	5 ± 2**	24 ± 9	44 ± 18**
HAv-SF-10 (orally)	326 ± 22	3223 ± 611	172 ± 145	5207 ± 639	5 ± 2	15 ± 3	0 ± 0	112 ± 43	23 ± 15	9690 ± 1457	219 ± 109	4642 ± 804

Data are mean ± SEM and statistical significance was analyzed by using ANOVA with a Bonferroni posttest. n = 4–5. $^{*}P < 0.05$, $^{**}P < 0.01$, vs HAv-SF-10(p.o.) HAv stimulation(+)

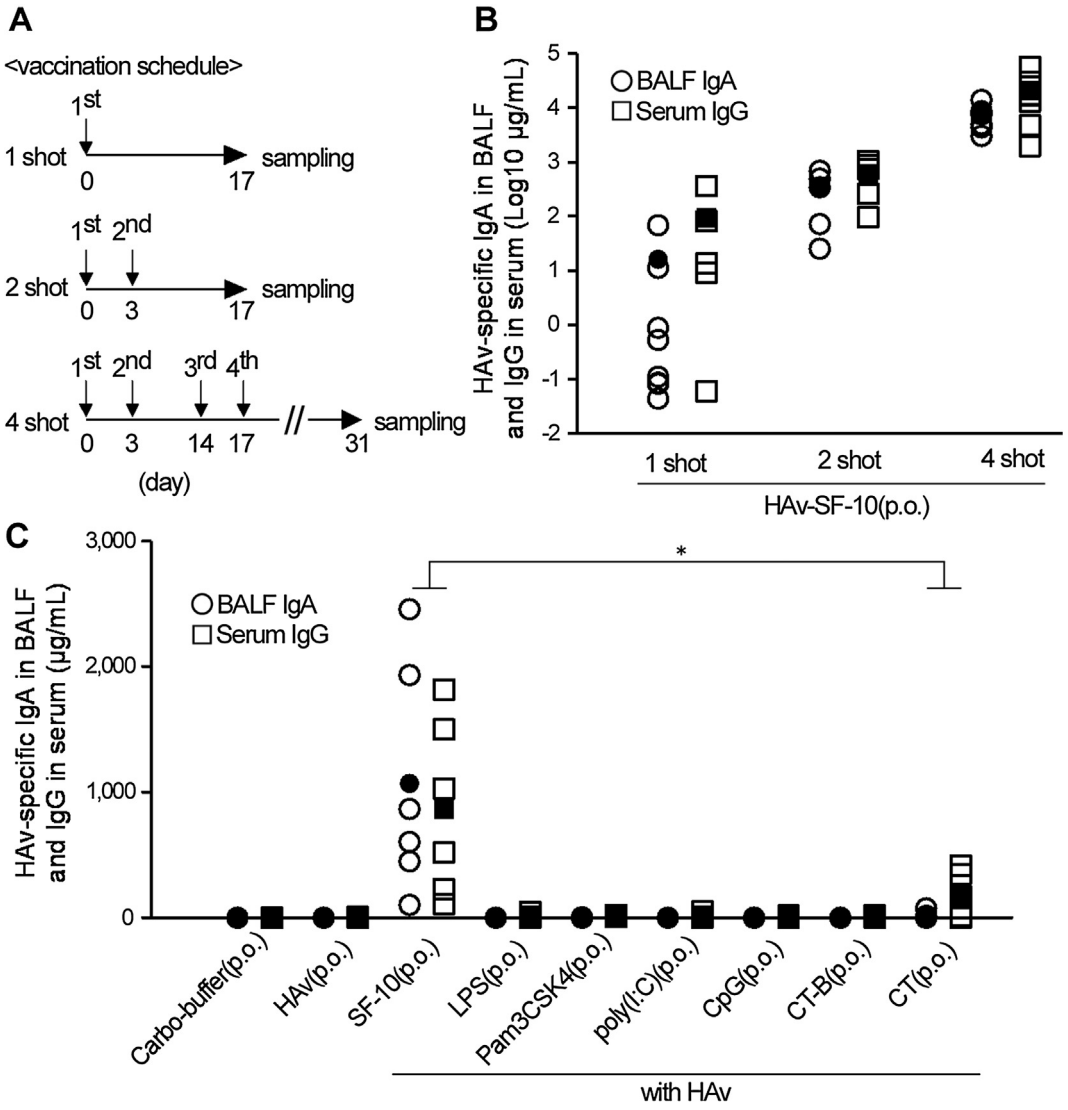

Fig. 4. Optimal time of immunization with HAv-SF-10(p.o.) and immunization efficacy of SF-10 compared with other mucosal adjuvants. Mice were immunized 1–4 times with HAv-SF-10(p.o.) according to the indicated schedule (A). At 2 weeks after the last immunization, the levels of HAv-specific S-IgA in BALF and HAv-specific IgG in serum were measured by ELISA (B). Mice were immunized twice at days 0 and 3 with HAv-SF-10(p.o.), or orally immunized with HAv combined with LPS, Pam3CSK4, poly(I:C), CpG, CT-B or CT. At 2 weeks after the last immunization, the levels of HAv-specific S-IgA in BALF and HAv-specific IgG in serum were measured by ELISA (C). Data represent HAv-specific S-IgA (circles) in BALF and HAv-specific IgG (squares) in serum of individuals (open) and their means (solid) (n = 5–6). Experiments were independently repeated two times with 5–6 mice per group and statistical significance was analyzed by using Welch's *t*-test. $^{*}P < 0.05$.

into gastrointestinal mucosal APCs. ii) Vaccination with HAv-SF-10 (p.o.) induced systemic and local HAv-specific antibodies in serum and various mucosal fluids, respectively, higher than those by HAv (s.c.), HAv-SF-10(i.n.) and HAv(p.o.) vaccinations. HI titers and protective immunity induced by HAv-SF-10(p.o.) against severe influenza A virus infection were higher than those induced by HAv-CT (p.o.). iii) Among the mucosal adjuvants tested in this study, which included adjuvants of microbial origins, SF-10 showed the highest adjuvanticity in gastrointestinal administration route. iv) Marked induction of both HAv-specific IgA/S-IgA and IgG antibody-producing cells in respiratory and gastrointestinal mucosal lymph nodes of mice immunized with HAv-SF-10(p.o.), compared with mice immunized with HAv(p.o.). v) Administration of HAv-SF-10 (p.o.) showed unique cytokine induction patterns in the spleen; highest induction of Th17, high induction of Th1 and moderate induction of Th2 cytokines.

We reported previously that pulmonary surfactant mimicking SF-10 adjuvant amplifies antigen uptake into mucosal APCs in the nasal cavity and induces humoral and cellular immunities [17]. The natural pulmonary surfactant, which is secreted from alveolar type II epithelial cells, is absorbed and metabolized rapidly not only in respiratory mucosa but also gastrointestinal mucosa cells [18–21,34]. The findings suggest that antigen complexed with SF-10 is incorporated efficiently into the gastrointestinal mucosa and APCs and then induces antigen-specific immunities. Indeed, we found that SF-10 enhanced antigen uptake into intestinal APCs, such as $CD11b^{+}CD103^{+}$ DCs and $CD11b^{+}CD103^{-}$ DCs, the major subsets of intestinal DCs for oral vaccination. These DCs primed with antigen migrate from the intestinal lamina propria to MLN and present antigen information to T cells [33]. Intestinal $CD11b^{+}$-$CD103^{-}$ DCs induce IFN-γ-and IL-17-producing effector T cells [35], and $CD11b^{+}CD103^{+}$ DCs induce the development of regulatory T cells and control antigen specific immune tolerance [36]. $CD25^{+}$-$Foxp3^{+}$ regulatory T cells play an important role in intestinal IgA production [37] and $CD11b^{+}CD103^{+}$ DCs also induce IgA production in the intestine [38]. Since HAv-SF-10(p.o.) vaccination

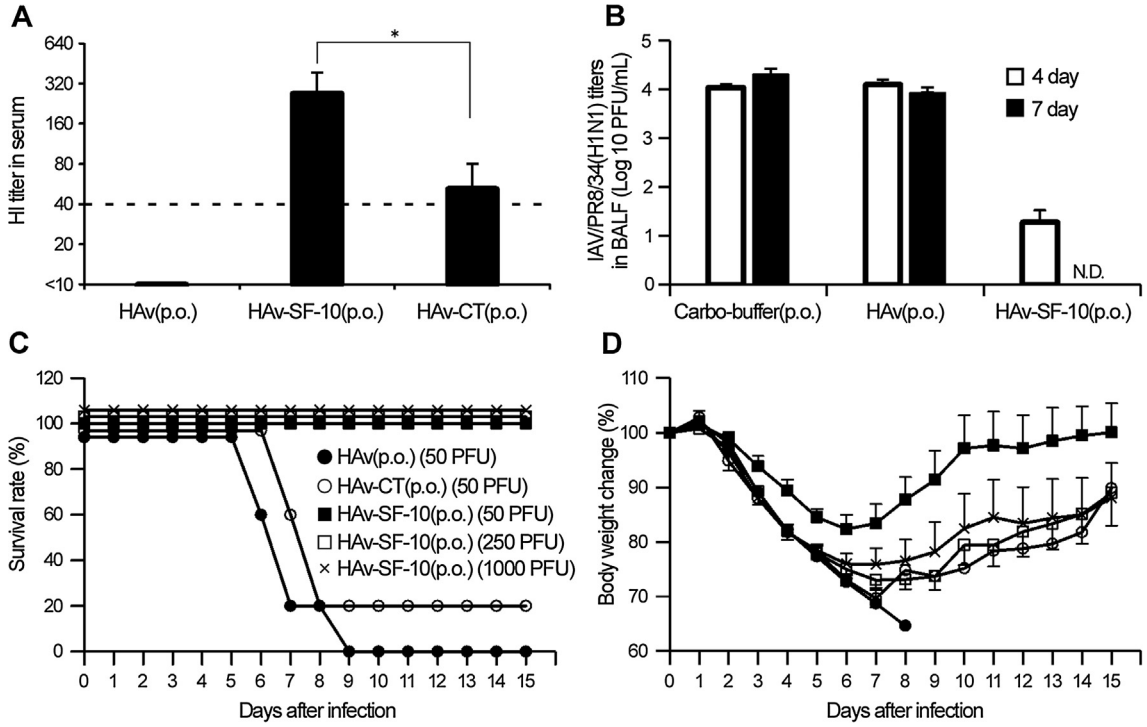

Fig. 5. Protective immunity induced by HAv-SF-10(p.o.) and survival rates of immunized mice after infection with lethal doses of IAV/PR8/34(H1N1). Mice were immunized orally with carbo-buffer, HAv, HAv-SF-10 or HAv-CT at days 0 and 3. HI titers were measured at 2 weeks after the last immunization. Data are mean ± SEM of 5–6 mice and statistical significance was analyzed by using Student's *t*-test. $^{*}P < 0.05$ (A). At 2 weeks after the last immunization, mice were infected with 50 × LD_{50} (50 PFU) of IAV/ PR8/34 (H1N1). Virus titers in BALF of immunized mice were measured at days 4 and 7 after infection. Data are mean ± SEM of 3–10 mice (B). At 2 weeks after the last immunization, mice were infected with 50 × LD_{50} (50 PFU), 250 × LD_{50} (250 PFU) or 1000 × LD_{50} (1000 PFU) of IAV/ PR8/34(H1N1), and survival rates of each group of mice (C) and body weight loss (D) were monitored for 15 days after infection (n = 4–5). Data of body weight loss are mean ± SEM of living 4–5 mice in each group during the experimental period. Significance of survival rates was determined by the Kaplan-Meier method. Experiments were independently repeated three times.

induced HAv specific systemic IgA and mucosal S-IgA very efficiently, SF-10 may stimulate the function of Th17 and regulatory T cells in the gastrointestinal tract. It has been reported that oral vaccination induces immune tolerance in general and, if anything, it is difficult to induce protective immunity efficiently by oral vaccination against infectious pathogens compared with other administration routes of vaccines, such as subcutaneous, intramuscular and intranasal administrations [39,40]. Efficient induction of protective immunity by HAv-SF-10(p.o.) vaccination in Fig. 5 may be derived from effective vaccine delivery across intestinal mucosal surface barriers and antigen presentation in the gastrointestinal mucosal APCs, because pulmonary surfactant, the base compound of SF-10, is absorbed by the intestinal mucosal epithelium and respiratory epithelium and metabolized rapidly and physiologically.

To develop an effective influenza vaccination, a simple and easy inoculation method for mass vaccination is required. Subcutaneous or intramuscular vaccination requires needles and syringes, and intranasal vaccination requires spray devices, whereas oral vaccination does not require any special device. In addition, seasonal human influenza virus infects the respiratory mucosa and highly pathogenic avian influenza viruses, such as H5N1, infect both respiratory and gut mucosa [8]. Thus, it is desirable to induce anti-influenza virus immunities in both the respiratory and gastrointestinal mucosae to prevent such infection. Based on the results of this study, we propose that oral administration of HAv-SF-10, which induced higher levels of systemic and mucosal HAv-specific antibodies compared with other vaccination routes (Fig. 2), and also induced HAv-specific ASCs in the respiratory and gastrointestinal mucosal lymphoid tissues (Fig. 3). Notably, the induced levels of HAv-specific serum IgG and BALF S-IgA and HI titers by HAv-SF-10(p.o.) were strikingly higher than those induced by HAv-SF-10(i.n.) and HAv(s.c.) (Fig. 2A and B, Table 1). These results suggest that the adjuvanticity of SF-10 is more effective on gastrointestinal mucosa than on nasal mucosa. In addition, the high levels of HAv-specific S-IgA in the gastrointestinal tract (Fig. 2D and E) and high amounts of HAv-specific ASCs in PP and MLN (Fig. 3D and E) of mice immunized with HAv-SF-10(p.o.), suggest that oral administration of HAv-SF-10 is potentially suitable for prevention of influenza virus infection.

Vaccination with HAv-SF-10(p.o) elicited HAv-responsive Th1, Th2 and Th17 cytokine-producing cells (Table 2). Th1 and Th2 cells are major subsets of CD4 helper T cells and modulate cellular and humoral immunity, respectively. In our previous study, immunization using HAv-SF-10(i.n.) elicited HAv-responsive Th1 type cytokine- (IFN-γ), and Th2 type cytokine (IL-4)-producing lymphocytes and induced antigen specific humoral and cell mediated immunity [12,17]. In the present study, we found markedly high HAv responsive IL-17A production levels in mice vaccinated with HAv-SF-10(p.o.) and HAv-SF-10(i.n.) compared with HAv(p.o.), HAv(i.n.) and HAv(s.c.). Recently, Th17, a new subset of T helper cells, was found to produce IL-17A and IL-22 [41] and was related to the development of antigen-specific IgA ASCs in PP [42]. SF-10 enhanced antigen uptake into intestinal $CD11b^{+}CD103^{-}$ DCs (Fig. 1), which induced IL-17A producing effector T cells. The finding implies that the induced Th17-mediated immunity is one of the mechanisms of efficient induction of mucosal S-IgA by HAv-SF-10 (p.o.), although the negative issues of Th17-mediated immune responses, such as hyperresponsiveness and inflammatory stimulation in the pathogenesis of the respiratory syncytial virus infection [43] and influenza virus infection [44], have been reported. Furthermore, immunization with HAv-SF-10(p.o.) induced mild levels of HAv-responsive IL-4 (Table 2), which induces IgE,

although marked induction of IL-4 was detected by HAv(s.c.). Indeed, HAv(s.c.) vaccination induced HAv-specific serum IgE [12] but the levels of IgE induced by HAv-SF-10(p.o.) were below the detection levels (data not shown).

It has been reported that bacterial- or viral-based mucosal adjuvants administrated through the intranasal and oral routes induce both mucosal S-IgA and serum IgG [45]. In the present study, mice were immunized orally twice with HAv combined with LPS, Pam3CSK4, poly(IC), CpG, CT-B, CT, or SF-10, in order to compare the adjuvanticity of SF-10 with these mucosal adjuvants. All adjuvants other than CT and SF-10 exhibited little or no adjuvanticity. While SF-10 induced both HAv-specific S-IgA in BALF and IgG in serum, CT induced HAv-specific IgG in serum only but not HAv-specific S-IgA in BALF (Fig. 4C). In general, the antigenicity of proteins administered orally degenerates in the low gastric pH environment and digestive enzymes in the gastrointestinal tract, making it difficult to induce effective oral immunization [40]. To improve antigen stability in gastrointestinal tracts, polymerized liposomes which are susceptible to be uptaken by Peyer's patches and provide superior protection against antigen degeneration have been reported [46]. Antigen combined with liposome-like adjuvant SF-10 in carbo-buffer may protect antigen degeneration in the gastrointestinal tract and induce effective systemic and local immunity compared with other mucosal adjuvants tested.

For protection against unexpected outbreaks of emerging infections, it is important to develop effective vaccines that can rapidly induce robust protective immunity. Immunization with HAv-SF-10 (p.o.) induced potent protective immunity with high HI titers and neutralization activities against severe IAV infection with a short period of time in mice (Fig. 5). Although HAv-CT(p.o.) induced HAv-specific IgG in serum with HI titers > 40 (Fig. 4C and 5A), sufficient levels of HAv-specific S-IgA were lacking in BALF and only 20% of the mice were alive after severe influenza virus challenge. While the above results highlight the superiority of HAv-SF-10(p.o.) with regard to the induced systemic and local immunity, compared with the other preparations used in our study, they also indicate that anti-HAv S-IgA levels in the lungs induced by HAv-SF-10 (p.o.) play pivotal roles in protection against IAV infection.

Our study has certain limitations. Oral vaccination shows in generally two reciprocate aspects, immune tolerance induction and protective immunity induction, although details of difference in the mechanisms between them have not been well analyzed. In the present study, we clarified the protective immunity induced by HAv-SF-10(p.o.) vaccination, but not yet the immune tolerance induction. In addition, we suggested that protective immunity induced by HAv-SF-10(p.o.) is associated with the marked induction of Th17 cytokines, high induction of Th1 cytokines and moderate induction of Th2 cytokines, although the negative issues of Th17-mediated immune responses, such as hyperresponsiveness and inflammatory stimulation in the pathogenesis of viral infection [43.44], have not been studied yet. To demonstrate the translational values of our new oral vaccine formulation, further studies are required. We are planning to clarify these limitations described above and cell-mediated immunity by HAv-SF-10(p.o.) in mice and non-human primates.

In conclusion, we have demonstrated in the present study that SF-10, which mimics human pulmonary surfactant, is a suitable and effective mucosal adjuvant that can be used for oral route vaccination to provide potent and rapid systemic and mucosal immunity.

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Conflict of Interest

The authors declare no conflict of interest.

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